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Diagnosis and Characterization of Insecticide-insensitive Acetylcholinesterase in Three Populations of the Sweetpotato Whitefly *Bemisia tabaci*

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Abstract: A biochemical approach was used to characterize acetylcholinesterase (AChE) insecticide insensitivity in several sweetpotato whitefly (*Bemisia tabaci;* SPW) populations. Discriminating doses of insecticide were established to differentiate between sensitive and insensitive SPW strains and to genotype individual whitefly. This technique was then used to examine the frequency of insensitive AChE alleles in several SPW populations and to isolate a line homozygous for insensitive AChE from a heterogenous B-type population. Inheritance of putative altered AChE genotypes was consistent with the proposed haplo-diploid status of *B. tabaci*. This biochemical diagnostic was also employed to determine the role of insensitive AChE in the observed resistance profiles of several laboratory populations subjected to different selection regimes. In keeping with previous studies on insecticide resistance in SPW, resistance does not appear to be uniquely associated with the B-type but rather with SPW populations found in crop systems. © 1998 SCI.

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Key words: acetylcholinesterase; whitefly; resistance; insecticide

1 INTRODUCTION

Over the past decade, the novel 'B-type' of the sweetpotato whitefly or SPW (*Bemisia tabaci* Genn.), has caused unprecedented economic damage to cotton and vegetable crops in the USA. Recent studies have described this novel 'B-type' of SPW as either a separate species, the silverleaf whitefly *Bemisia argentifolii* Bellows & Perring,[1] or as part of a proposed *Bemisia* species complex.[2] One of the most important factors contributing to recent severe outbreaks of this pest has been the appearance of high levels of resistance to many of the insecticides currently employed in whitefly control.[3–6] For example, recent studies in Arizona SPW populations have indicated that significant resistance can be found to endosulfan, synthetic pyrethroids, organophosphorus and carbamate insecticides.[7]

Resistance management programs are often implemented with little knowledge of the resistance mechanisms present in the pest insects or their specific frequencies in different populations. In the past this has

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also been the case for strategies aimed at controlling resistant SPW. A knowledge of the principal resistance mechanisms responsible for field control failures, coupled with a means to monitor for resistance genotypes in individual insects can provide a platform for two essential processes in resistance management to be carried out: (1) the detection of resistance at sufficiently low frequency to allow evasive action to be taken before control failures occur and (2) the rational design of resistance management strategies incorporating compounds to which existing resistance mechanisms do not confer cross-resistance. Both these considerations become especially relevant when strategies involving mixtures or alternations of different chemical control methods are planned or are already in place. For example, if the choice of pesticide is limited by multiple resistance, as is the case for SPW in the state of Arizona, USA,[7] it is essential that susceptibility to existing effective compounds be preserved through extensive resistance monitoring.

Until the recent introduction of insect growth regulators, organophosphorus and carbamate insecticides have been the two most widely used chemical control agents in whitefly control and are likely to remain important in alternation or rotation strategies with such novel compounds. The primary site of action for both organophosphorus and carbamate insecticides is acetylcholinesterase (AChE).[8] This enzyme plays a key role in terminating excitatory neurotransmitter action in insect synapses.[9] Insensitive forms of AChE have been found in a wide range of insects from different orders.[10,11] Two insensitive AChE variants have previously been documented in a number of SPW populations, predominantly classified as B-type. Each form shows a differential insensitivity to the organophosphorus insecticides paraoxon and azamethiphos.[4]

The central aim of this study was therefore to characterize AChE insecticide insensitivity at a biochemical level with reference to susceptible and resistant SPW populations from North and Central America and to use these data to design biochemical diagnostics capable of discriminating between putative AChE genotypes. These diagnostics were then employed to: (1) examine the frequency of insensitive AChE in several whitefly populations, (2) isolate a resistant line homozygous for insensitive AChE and (3) investigate the role of insensitive AChE in several SPW populations subjected to different selection regimes in the laboratory.

2 MATERIALS AND METHODS

2.1 Insect populations

Three strains of *Bemisia tabaci* were used to derive diagnostic doses for discriminating between putative insensitive AChE genotypes. These originated from three different source populations, two from North America, A_1 (Sinaloa, Mexico) and B (Arizona, USA), and one from the Caribbean basin (Puerto Rico). Each population shows a unique non-specific esterase banding pattern that can be used as a diagnostic to distinguish these and other putative populations.[12] The strain from Puerto Rico comes from a SPW population found exclusively on a single non-crop host plant, *Adenoropium* (*Jatropha*) *gossypifolium* (L.) Pohl. (JAT population), and is therefore unlikely to have been exposed to insecticide selection. The A_1 and B colonies used in this study were collected from host plants located within intensively sprayed agricultural crops. Colonies were established in the laboratory on the same host plants from which the SPW were collected and maintained in culture at the Department of Plant Sciences, University of Arizona-Tucson. Esterase profiles of laboratory cultures were routinely assessed to verify the esterase electromorph distinguishing each population.

For insecticide selection experiments, subsets of a B-type colony were reared on cotton plants and were either left unselected (C), or selected with the pyrethroid fenpropathrin (F) ('Danitol', (*RS*)-α-cyano-3-phenoxy benzyl 2,2,3,3-tetramethylcyclopropanecarboxylate, 92·5%; Valent USA Corporation, Walnut Creek, CA) or a 5 : 1 mixture of fenpropathrin and the organophosphorus insecticide acephate (FA) ('Orthene', *O*,*S*-dimethyl acetylphosphoramidothioate, 99·6%; Valent USA Corporation, Walnut Creek, CA). In addition, one population selected with the fenpropathrin + acephate mix was subsequently left unselected for six generations (FAR) in order to examine whether resistance levels declined in the absence of selection. Details of the colony rearing and selection regimes applied are described in detail elsewhere.[13] Bioassays were conducted using the glass-vial technique as previously described.[14] Briefly, experiments were replicated three times and chemicals were applied by adding 225 μl of insecticide in acetone into each vial. Each vial was then rotated until the acetone had evaporated, leaving the interior of each vial coated evenly with the chemical residue. Control vials coated with acetone alone were also tested. Twenty to thirty adult whitefly were transferred by mouth aspiration to the treated vials and were held at 20 ± 2°C for 3 h before being scored for mortality. Two lines of whitefly from the original founding colony were selected over 13 generations using either the fenpropathrin or fenpropathrin + acephate treatment. After the sub-lines were subjected to these different selection regimes, a series of six to eight doses of the fenpropathrin or fenpropathrin + acephate insecticide mix were used to estimate LC_{50} values and resistance ratios for each line. Table 1 provides a summary of all whitefly populations used in this study and the LC_{50} values for each strain.

TABLE 1

Name	*Source*	*Laboratory selection*	*Fenpropathrin* LC_{50} (*μg/vial*)	*Fenpropathrin + acephate* LC_{50} (*μg/vial*)
JATROPHA (JAT)	Puerto Rico	—		
A_1	Mexico	—		
B	Arizona	—		
C	Arizona	Control for selected lines below	0·29	0·20
F	Arizona	Danitol	3·43	0·21
FA	Arizona	Danitol-Orthene	217	248
FAR	Arizona	Danitol-Orthene, then no selection for six generations	180	196

2.2 AChE activity

Biochemical assay of AChE activity was carried out according to the method of Ellman *et al.*[15] using acetylthiocholine (ASCI) as the artificial substrate. Dose-dependent inhibition of AChE was estimated using a range of concentrations of representative carbamate (carbofuran, propoxur) and organophosphorus (paraoxon, methamidophos) insecticides. Initial studies on mass homogenates were conducted to establish the overall level of AChE insensitivity in the three populations (JAT, A_1 and B) to carbamate and organophosphorus inhibition. Data from these initial studies were subsequently used to derive diagnostic doses capable of discriminating between putative sensitive and insensitive AChE genotypes in individual insect assays, as previously described.[4,16]

Mass homogenates of whitefly were prepared by homogenizing adults in 0·1 M phosphate buffer containing 1 g litre^{-1} 'Triton' X-100. The equivalent of one-third of a whitefly was used as an enzyme source for inhibition studies. Individual whitefly were homogenized in a 96-well microtiter plate containing 70 μl of 'Triton'-X100/phosphate buffer using a multiple homogenizer.[17] AChE activity was assayed for each individual by pipetting equal fractions of the homogenate into three wells of a separate microtiter plate. Uninhibited and inhibited activity were monitored in a kinetic microtiter plate reader (Thermo$_{max}$, Molecular Devices). Biochemical assays were performed at room temperature and graphs of reaction rates were plotted for each of the 96 wells. By measuring reaction rates in the presence of an insecticide dose designed to discriminate between sensitive and insensitive forms of AChE and by comparing these reaction rates with those in the uninhibited controls, it was possible to ascribe putative genotypes to individual insects. Populations of whitefly deemed to be homozygous susceptible (*S*/*S* females or putatively *S*/− haploid males) or homozygous resistant (*R*/*R* females or putatively *R*/− haploid males) were defined by calculating prediction interval limits for the mean percentage of activity remaining at a discriminating dose of insecticide. After checking for normality using the Kolmogorov–Smirnov (Lilliefors) test (SYSTAT 6.0 for Windows, SPSS Inc., Chicago, IL, 1996), prediction intervals were determined using the following equation:

$$\bar{x} \pm t_{\alpha, n-1}\sqrt{s^2}\sqrt{\frac{n+1}{n}}$$

where $t_{\alpha, n-1}$ is a T distribution with $\alpha = 0{\cdot}10$ and $n - 1$ parameters. Under these conditions, there is a 90% chance that any new value drawn from the same distribution will fall within prediction intervals defined for either homozygous susceptible or resistant populations. Heterozygous individuals possessing intermediate levels of remaining activity were differentiated by their failure to fall into either of the predicted intervals for homozygous susceptible or resistant genotypes. Although values for the JAT and A_1 populations appeared to be normally distributed, in testing for normality, one individual outlier from the A_1 female population (marked with an asterisk in Fig. 3b) was removed from the dataset.

The catalytic properties of the SPW AChE enzyme were estimated by measuring reaction rates (V) over a range of substrate (S) concentrations. Eadie-Hofstee plots (V versus V/S) were constructed to determine the K_m (Michaelis constant) and V_{max} (maximum turnover rate) values for each population. Estimates of IC_{50} values for a representative carbamate (carbofuran) and organophosphorus (paraoxon) insecticide were derived from inhibition studies on each whitefly population.

3 RESULTS

Preliminary dose-inhibition studies on mass homogenates demonstrated that AChE activity in the JAT

population was sensitive to inhibition by both carbamate insecticides and the organophosphorus insecticide paraoxon (Fig. 1a–c). In contrast, both the Mexico A_1 and Arizona B populations were relatively insensitive to these insecticides. However, methamidophos had very little effect on AChE activity in any of the populations examined in this study (Fig. 1d). In the insecticide-selected whitefly lines, levels of AChE insensitivity did not differ between the reference control and the fenpropathrin or fenpropathrin + acephate laboratory-selected populations (data not shown). Therefore, although all populations derived from these laboratory-selected lines appear to carry insensitive AChE, this mechanism alone cannot account for the differences in resistance observed between fenpropathrin- or fenpropathrin + acephate-selected populations.

Discriminating doses capable of ascribing putative genotypes to individual insects were derived for both carbofuran (3 μM) and paraoxon (30 μM) from the initial inhibition studies illustrated in Fig. 1. Thus, a dose was chosen that gave maximum discrimination between the inhibition curves for the different populations (see dashed lines in Fig. 1a and c). Carbofuran was selected for subsequent analysis of individual whitefly as this insecticide gives the best discrimination between putative genotypes (Fig. 1a). In the susceptible JAT strain, the discriminating dose of this insecticide gave an almost complete inhibition of AChE activity ($15 \pm 1{\cdot}76\%$ of uninhibited activity). In contrast, activity levels in the reference resistant strain (A_1) were left unaffected at this concentration of carbofuran ($101 \pm 5{\cdot}9\%$ of uninhibited activity) whereas an intermediate level of activity was observed for the relatively heterogenous B-type population ($64 \pm 1{\cdot}73\%$ of uninhibited activity). This discriminating dose of carbofuran, estimated from mass homogenates, was then used to distinguish putative AChE genotypes in individual whitefly assayed from each reference population (JAT, A_1 and B) and from several single-family lines (B-cross) derived from the original B-type population.

Analysis of individual insects from different populations showed that all JAT individuals were highly sensitive to inhibition by diagnostic doses of either carbofuran or paraoxon (Fig. 2a). In contrast, the A_1 strain appeared uniformly homozygous for insensitive AChE (Fig. 2b), whereas the Arizona B strain contained a mixture of putative genotypes (Fig. 2c). The biochemical assay adapted for individual whitefly was also used to isolate a line homozygous for insensitive AChE from the originally heterogenous B strain (Fig. 2d).

Interestingly, in the analysis of individual whitefly, males always appeared as either completely sensitive or

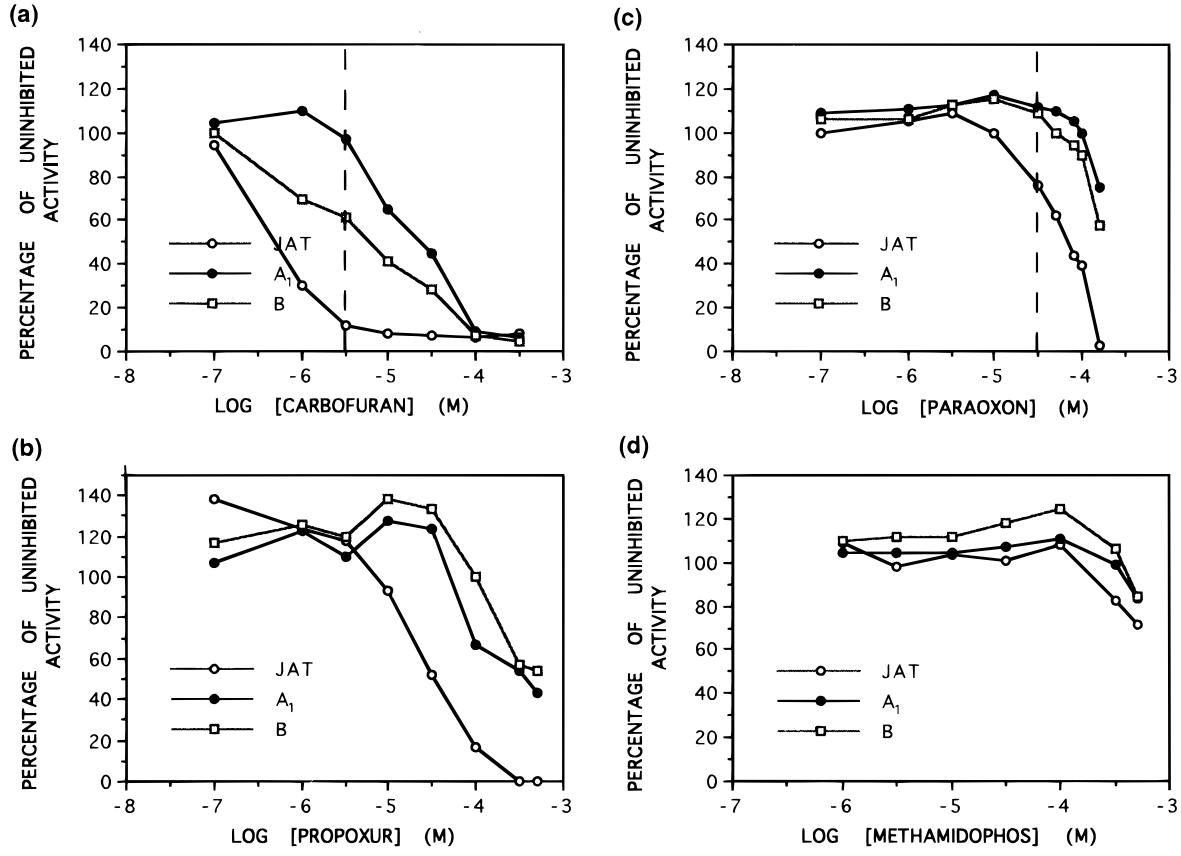

Fig. 1. Dose-dependent inhibition of AChE activity in reference susceptible (JAT) and resistant (A_1 and B) whitefly populations. Insecticide concentrations used to discriminate between putative genotypes in subsequent whitefly assays are indicated by a dashed line.

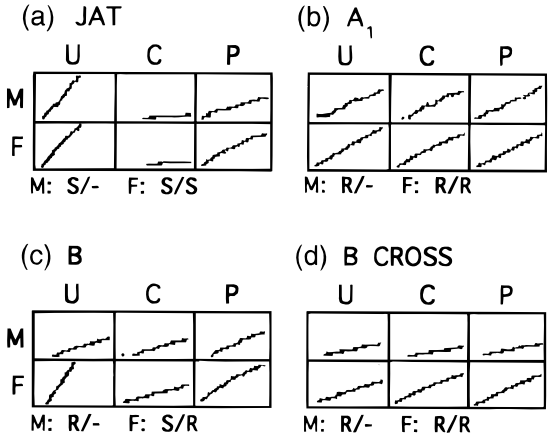

Fig. 2. Biochemical genotyping of insensitive AChE genotypes in individual whitefly from three reference populations: (a) JAT, (b) A_1, (c) B and (d) progeny from a single-pair mating of the original B-type population. Males (M) and females (F) are shown separately. Treatments were U, uninhibited; C, 3 μM carbofuran and P, 30 μM paraoxon. AChE activity in each well is determined by measuring change in absorbance (Y axis, 0·05 OD limit) with time throughout the duration of the assay (X axis, 30 min).

insensitive AChE activities, consistent with their presumed haploid status (where males are either $S/-$ or $R/-$) and never appeared heterozygous (R/S). In contrast, only females exhibited all three putative genotypes (S/S, R/S or R/R), reflecting their presumed diploid condition. Results from the analysis of all individual whitefly assayed were also displayed as histograms (Fig. 3). For each individual, AChE activity remaining in the presence of a diagnostic dose of carbofuran is expressed as a percentage of total uninhibited activity. Examination of the histograms in Fig. 3 shows that putative S/S or $S/-$ genotypes (Fig. 3a) can be clearly discriminated from putative R/R or $R/-$ genotypes (Figs 3b,d). In addition, whereas only B males appear to display either $S/-$ or $R/-$ profiles, a small proportion of B females possess an intermediate putative R/S genotype, showing carbofuran-inhibited AChE activities outside of the predicted intervals for either S/S or R/R genotypes (Fig. 3c). The absence of susceptible whitefly in the assayed progeny derived from a single-pair cross from the B population (Fig. 3d), compared to data from the original heterogenous B-type population (Fig. 3c), suggests that this single family was established from a hemizygous and homozygous resistant male and female respectively ($R/-$ male × R/R female). The resulting sub-line therefore appears homozygous for resistance.

Uninhibited AChE activity was reduced in both resistant (A_1 and B) populations compared to that observed in the JAT population. This observation is consistent with previous work indicating that target-site resistance is associated with a change in the kinetic properties governing substrate hydrolysis.[11] Differences in K_m and V_{max} were also observed between the homozygous susceptible (JAT) and resistant (A_1 and B cross) populations (Fig. 4). The kinetic properties of the homozygous resistant line derived from a single-pair cross between parents of the original B population appeared indistinguishable from the resistant A_1 population (Fig. 4). In both resistant populations, the K_m value was three-fold greater than in the JAT population, reflecting a marked decrease in the affinity of the insensitive enzyme. V_{max} was also affected in both the homozygous resistant A_1 population and in the progeny derived

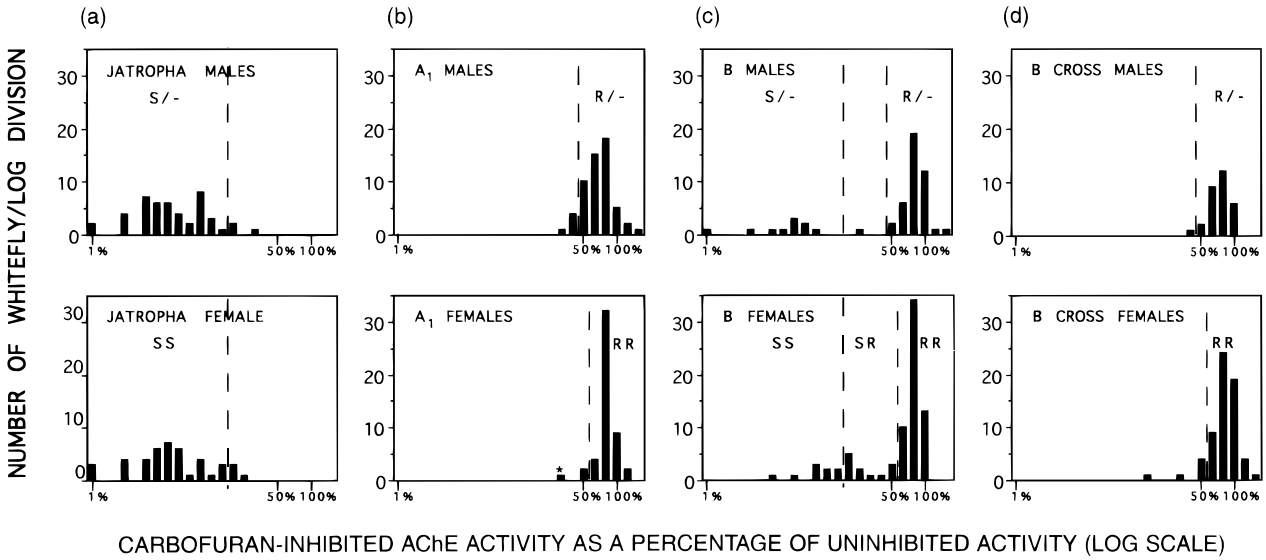

Fig. 3. Histograms of carbofuran-inhibited AChE activity expressed as a percentage of uninhibited activity in the same individual whitefly. Whitefly populations are as follows: (a) JAT, (b) A_1 and B, (c) before and (d) after single family selection for homozygosity. Note the disappearance of susceptible whitefly following selection (d). Distributions for males (upper panels) and females (lower panels) are shown separately. The log of AChE insensitivity to insecticide inhibition is plotted on a linear scale and predicted distribution intervals for homozygous susceptible and resistant populations are indicated by dashed lines in the histogram plot. *, Point not included in the Kolmogorov–Smirnov (Lilliefors) test.

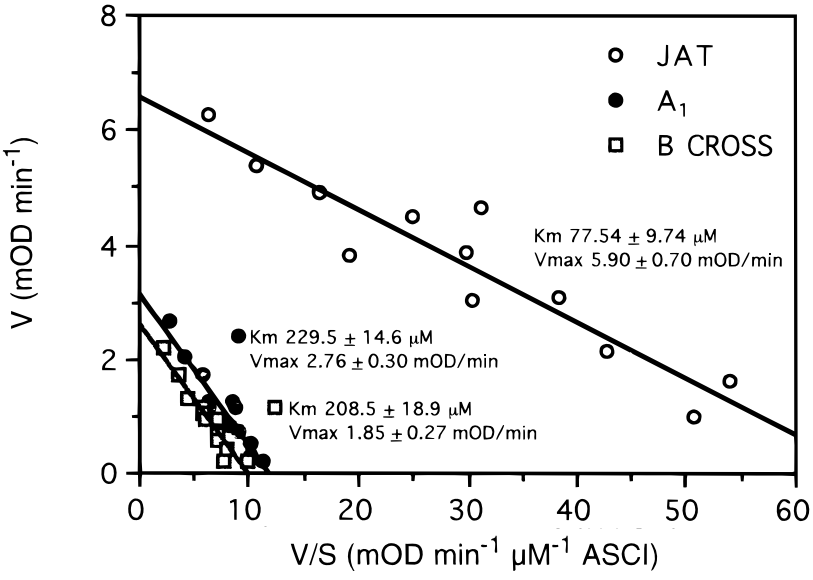

Fig. 4. Representative Eadie–Hofstee plot of AChE activity (V) over a range of substrate concentrations (V/S) for homozygous sensitive (JAT), insensitive (A_1) and B-cross progeny (B cross) derived from a single-pair cross from the original B-type population. K_m and V_{max} values for each population represent the mean (±S.E.M.) of five independent experiments.

from the B cross. Inhibition studies demonstrated that the sensitivity of the homozygous susceptible (JAT) population to increasing concentrations of carbofuran and paraoxon was 50- to 70-fold greater than for both the resistant (A_1) and B cross progeny (B). Thus the IC_{50} value for carbofuran in the JAT population was 1·17 μM (±0·49 S.E.M.), and the corresponding IC_{50} values for the A_1 and B cross progeny were 70·3 μM (±8·72 S.E.M.) and 55·9 μM (±9·96 S.E.M.), respectively. In the case of paraoxon, IC_{50} values could only be accurately estimated in the JAT population (88·1 μM ± 20·5 S.E.M.) as the highest permissible concentrations of this insecticide failed to inhibit more than 50% activity in either A_1 or B cross populations.

4 DISCUSSION

We were interested in (1) developing a biochemical diagnostic for insensitive AChE in American populations of SPW capable of discriminating between putative genotypes; (2) investigating the role of insensitive AChE in a series of whitefly lines subjected to different selection regimes; (3) examining differences in the kinetic properties of sensitive and insensitive forms of AChE in the whitefly populations selected for this study.

Individual whitefly assays consistently demonstrated that males were always either fully susceptible or resistant to insecticide inhibition whereas some females appeared to possess an intermediate profile, indicative of heterozygozity. These results are in agreement with a recent biochemical study providing evidence of apparent (phenotypic) haplodiploidy in SPW.[18] Thus males are presumed to be always hemizygous for resistance due to their haploid condition (putatively $S/-$ or $R/-$). The presumed haplo-diploid status of SPW is important for two reasons. Firstly, single copies of resistance genes expressed in hemizygous males ($R/-$) will behave like resistant homozygotes (R/R) under selection and will survive higher doses of insecticide than heterozygous females (R/S), although both insects carry only a single copy of the resistant allele. Secondly, if SPW indeed exhibits haplodiploidy or functional haplodiploidy (whereby males and females are actually diploid but the paternally inherited genome is not passed on by males),[19] sex ratios in field populations of SPW could play a crucial role in determining effective resistance levels.

With respect to changes in the kinetic properties of the enzyme, both differences in K_m and V_{max} were observed between susceptible and resistant populations. Similar changes in the kinetic properties of the insensitive enzyme have also previously been described for AChE in other insecticide-resistant insect species.[11] Since the acetylation rate appears to be the limiting step in substrate hydrolysis in insects,[20] it is likely that the decrease in K_m observed in both homozygous resistant A_1 and B cross populations reflects a change in the substrate affinity of the enzyme.

The results from this study indicate that AChE is a widespread mechanism of resistance in North American SPW populations and that target-site insensitivity to OPs and carbamates is not restricted to the B-type alone. As previously reported from work on cyclodiene resistance associated mutation(s) in the GABA receptor subunit gene *Rdl* as well as biochemical and toxicological studies,[4,16,21,22] insecticide resistance does not appear to be uniquely associated with the B-type but rather appears to be associated with SPW populations

residing within crop systems and thus exposed to insecticides. Therefore, as we have previously speculated for resistance to cyclodienes, insecticide resistance does not appear to have acted as a unique force in the rapid spread of the B-type of *Bemisia tabaci.*

The SPW resistance management strategy in place in the US has promoted the use of mixtures (i.e. pyrethroids + organophosphorus insecticides) such as those employed in the selection regimes described in this paper. However, such management strategies are often implemented without any clear knowledge of the mechanisms underlying resistance. As SPW appears to be multiply resistant to many of the available compounds employed in its control, it is crucial to determine which of the available compounds selects *least* for resistance and to deploy effective pesticide mixtures appropriately.[23] In this respect, the combination of the pyrethroid fenpropathrin and the organophosphorus insecticide acephate has been widely used as a method of chemical control for the whitefly in the US, giving higher levels of toxicity than either compound alone.[7] However, both field and laboratory data suggest that the use of this mixture may lead to a rapid selection for resistance to fenpropathrin + acephate as well as lead to cross-resistance to other pyrethroid insecticides (Sivasupramaniam and Watson, pers. comm.).[7,23] For example, whiteflies selected in the laboratory with the fenpropathrin + acephate mixture developed over 1000-fold resistance to both the (5 : 1) mixture and to fenpropathrin alone in as little as 10 generations. In contrast, selection with only fenpropathrin showed only slight (5-fold) resistance over the same period to the mixture and no resistance to fenpropathrin alone.[13]

In view of the fact that the insecticidal efficacy of acephate alone is very low in most US SPW populations, there are two possible alternative explanations for these observations: either there is a lack of intrinsic sensitivity of whitefly AChE to methamidophos (the bioactivated form of acephate) or there is widespread target-site resistance to this compound. Our *in-vitro* biochemical assays using methamidophos as an AChE inhibitor in the whitefly, described here, confirm the high level of insensitivity of the whitefly enzyme to this organophosphorus compound (Fig. 1d). Thus, the combination of acephate and fenpropathrin is probably a synergistic one, in that acephate (or its bioactivated form) is virtually devoid of intrinsic insecticidal activity. The question of the inferred mode of action of acephate therefore becomes critical.

Although acephate is commonly viewed as an AChE inhibitor, our results suggest that its synergistic activity may result from a different mechanism. As assays of AChE sensitivity in the unselected, fenpropathrin + acephate and fenpropathrin selected strains were indistinguishable, the > 1000-fold resistance to this mixture generated in the selection experiments does not appear to involve selection for altered AChE. Our working hypothesis is therefore that methamidophos may act indirectly as an inhibitor of another esterase, most likely a pyrethroid-degrading esterase. The selectivity of methamidophos as an esterase inhibitor commonly shows a three to four order of magnitude preference for non-AChE enzymes.[24] This suggests that synergism may be caused by inhibition of esterase-mediated degradation of fenpropathrin. However, the very high resistance to the fenpropathrin mixture, as well as to fenpropathrin alone, in the fenpropathrin + acephate selected strain still requires further explanation.[7] An alternative hypothesis may therefore be that over-expression of a pyrethroid-degrading esterase provides resistance to both fenpropathrin alone and to the fenpropathrin + acephate mixture. Clearly this phenomenon warrants further investigation.

The AChE gene has been cloned from several insect species including *Drosophila melanogaster* Meig.,[25] two mosquito species[26,27] and the Colorado potato beetle *Leptinotarsa decemlineata* Say.[28] Recent molecular studies have identified several resistance-associated mutations within the acetylcholinesterase gene from *D. melanogaster*[29] and the house fly *Musca domestica* L.[30] However, molecular studies of the AChE gene from *B. tabaci* are still lacking. It is therefore unclear whether the same, or similar, point mutations underlying insensitivity in *D. melanogaster* and the house fly are found in the strains discussed here. The use of biochemical diagnostics described in this paper to derive insect lines homozygous for insensitive AChE represents an important first step towards the molecular cloning and analysis of the point mutations underlying AChE insensitivity, as well as providing a mechanism of measuring the frequency of specific resistance alleles in the field.

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